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Abbreviations : SI: sucrase isomaltase, L-PK: liver-pyruvate kinase, SGLT1: sodium glucose transporter, GPCR: G protein-coupled receptor, T1R: Taste Receptor type 1, 3OMG: 3-O-methyl glucose, N-AGA: N-acetylglucosamine, G-6-Pase : glucose-6-phosphatase, EGFP: enhanced green fluorescent protein, ChREBP: carbohydrate response element binding protein, ChoRE: carbohydrate response element, SREBP: sterol response element binding protein, LXR: liver X receptor, PKA: protein kinase A, ADRP, adipose differentiation-related protein.

ABSTRACT

Sugar consumption and subsequent sugar metabolism are known to regulate the expression of genes involved in intestinal sugar absorption and delivery. Here we investigate the hypothesis that sugar-sensing detectors in membranes facing the intestinal lumen or the bloodstream can also modulate intestinal sugar absorption. We used wild-type and GLUT2-null mice, to show that dietary sugars stimulate the expression of sucrase-isomaltase (SI) and L-pyruvate kinase (L-PK) by GLUT2-dependent mechanisms, whereas the expression of GLUT5 and SGLT1, did not rely on the presence of GLUT2. By providing sugar metabolites, sugar transporters, including GLUT2, fuelled a sensing pathway. In Caco2/TC7 enterocytes, we could disconnect the sensing triggered by detector from that produced by metabolism, and found that GLUT2 generated a metabolism-independent pathway to stimulate the expression of SI and L-PK. In cultured enterocytes, both apical and basolateral fructose could increase the expression of GLUT5, conversely, basolateral sugar administration could stimulate the expression of GLUT2. Finally, we located the sweet-taste receptors T1R3 and T1R2 in plasma membranes, and we measured their cognate Galpha Gustducin mRNA levels. Furthermore, we showed that a T1R3 inhibitor altered the fructose-induced expression of SGLT1, GLUT5 and L-PK. Intestinal gene expression is thus controlled by a combination of at least three sugar-signalling pathways triggered by sugar metabolites and membrane sugar receptors that, according to membrane location, determine sugar sensing polarity. This provides a rationale for how intestine adapts sugar delivery to blood and dietary sugar provision.

INTRODUCTION

Intestinal sugar delivery depends on the levels of expression of dissacharidases (i.e. sucrase-isomaltase, SI) and sugar transporters. Indeed, dietary glucose is transported across the apical membrane of enterocytes by the energy-dependent sodium-glucose co-transporter 1 (SGLT1) and dietary fructose by the facilitative transporter GLUT5 (Levin, 1994). At the basolateral membrane, glucose and fructose exit the intestinal epithelia mainly via GLUT2-dependent facilitated diffusion (Levin, 1994) to reach the bloodstream. In GLUT2-null mice, glucose delivery seems to be mediated via vesicular trafficking (Stumpel et al., 2001) and the mechanism by which fructose reaches the blood stream is probably dependent on the presence of GLUT5 in the basolateral membrane (Blakemore et al., 1995). In the case of a sugar rich meal, GLUT2 can be recruited into the apical membrane where it complements SGLT1 and GLUT5 transport capacities (Kellett and Brot-Laroche, 2005). Furthermore, the small intestine adapts to repeated sugar ingestion by increasing transporter expression over the course of a few days (Burant and Saxena, 1994; Goda, 2000; Inukai et al., 1993). Thus, the regulation of the transcription, expression and location of sugar transporters organizes sugar absorption. In pathological conditions this regulation can amplify sugar delivery creating a vicious circle. Indeed, in rat, streptozotocin-induced diabetes increases SGLT1, GLUT2 and GLUT5 mRNA levels and sugar absorption (Burant et al., 1994; Ferraris et al., 1993), possibly causing exacerbated postprandial blood glucose excursion. Knowledge of the mechanisms of sugar sensing at the luminal and basolateral enterocyte membranes is thus essential for an understanding of the mechanism of induction of sugar-sensitive genes.

The regulation by sugars of gene expression is highly conserved through evolution, and is found in bacteria (Jacob and Monod, 1961), yeast (Johnston, 1999), plants (Villadsen and Smith, 2004), and mammals (Girard et al., 1997; Towle, 2005). In pancreatic ß cells, glucose is known to alter the expression of more than 150 genes that can be grouped in functional clusters, including insulin secretion, energy metabolism, membrane transport, signalling pathways, gene transcription and protein synthesis/degradation (Schuit et al., 2002). Fructose is known to regulate 50 genes in the small intestine of neonate rats, where it affects genes encoding for ion and hexose-transporters as well as enzymes of hexose metabolism (Cui et al., 2004).

Considering the number and functional variety of sugar regulated genes, and given their location in diverse sugar-sensitive tissues, a single signalling pathway is unlikely to explain

all cellular adaptations to life in a sugar-containing environment. Our current understanding of how cells respond to sugar is mainly based on studies in yeast and involves signalling pathways either initiated from hexose metabolites directly or from the direct engagement of hexoses with their cognate sugar receptors at the cell surface (Holsbeeks et al., 2004). In mammals, studies have been primarily focused on glucose metabolism and related signals (Girard et al., 1997; Towle, 2005). However, non metabolisable sugar analogues can stimulate SGLT1 expression (Miyamoto et al., 1993). Some sugar receptors have been identified in the plasma membrane of mammalian cells. A first group of mammalian sugar receptors is composed of sugar transporters, which can also function as sugar detectors. This group includes GLUT2 and SGLT3. Indeed, in hepatocytes GLUT2 can fuel sugar metabolism and also trigger a receptor-dependent protein signalling cascade (Guillemain et al., 2000; Guillemain et al., 2002). SGLT3 functions in cholinergic neurons neighboring enterocytes and while it does not transport glucose, it induces membrane currents upon sodium-dependent glucose binding (Diez-Sampedro et al., 2003). A second group of sugar detectors contains members of the G Protein–Coupled Receptor (GPCR) family. Among them are the sweet taste receptors of type 1, T1R, located on the tongue. Heterodimeric T1R3/T1R2 and T1R3 homodimers form sweet taste receptors that bind fructose or sucrose, leading to adenylate-cyclase signalling cascades (Nelson et al., 2001; Xu et al., 2004; Zhao et al., 2003). Biochemical data suggest that GPCRs participate to sugar signalling in an enteroendocrine cell line, but their molecular identification has yet to be determined (Rozengurt, 2006). In enterocytes, cell polarity further complicates the regulation of sugar sensitive genes. Indeed, detectors may conceivably be activated by dietary sugars at the apical membrane and by blood glucose at the basolateral membrane of enterocytes.

In this study we consider both metabolism-driven sugar signalling and sugar detection initiated signalling pathways. By monitoring the induction of sugar sensitive genes both *in vivo* and in Caco-2/TC7 cells we report here the identification of distinct sugar sensing signalling pathways with a striking polar distribution in enterocytes. These sugar sensory mechanisms offer insight not only into the modes of sugar absorption and delivery, but also unveil important avenues for the development of novel pharmacological compounds for improved glycaemic control.

MATERIALS AND METHODS

Mice: Wild-type mice were from C57Bl/6 strain (Janvier, France). GLUT2-null mice (RIP GLUT1XGLUT2-/-) (Guillam et al., 1997) were bred in the transgenic animal facility of IFR58 (Paris, France). All animal procedures are complied with recommendations for the use of laboratory animals from the French administration. Mice were fed for 5 days with the experimental diets, which contained either low amounts of sugar, or 65% (W/W) glucose or fructose as previously described (Gouyon et al., 2003a). The jejunum is here defined as the part of the small intestine starting at the Treitz ligament and excluding its last third. Intestinal samples, taken from fed mice, were rapidly everted and contents washed out in ice cold PBS before mucosa scrapings were taken. Mucosa was dispersed in RNA extraction buffer and snap frozen in liquid nitrogen.

Cell culture: Caco-2/TC7 cells were seeded at 6 x10^5 cells/cm^2 either on six-well, solid or porous (3µm high pore density) supports (Becton Dickinson, Meylan, France). Cells were grown in complete Dulbecco's modified Eagle's medium (25 mmol.L^{-1} glucose DMEM, Gibco, Paisley, U.K) supplemented with 20% heat-inactivated (30min, 56°C) fetal calf serum (FCS) (AbCys, Paris, France). Media were renewed every 24 hours for at least 10 days to allow differentiation of the cells (Chantret et al., 1994; Mahraoui et al., 1994).

Post-confluent, differentiated cells were switched from standard growth media (DMEM 25 mmol.L^{-1} glucose) to glucose-free DMEM supplemented with 10% heat-inactivated FCS, and contained less than 1 mmol.L^{-1} glucose (low sugar medium). According to need, media were supplemented with sugar for 2 to 4 days as indicated in the legend of the figures. For sugar metabolim assay; 25 mmole.L^{-1} 3-O-Methylglucoside or 250 mmole.L^{-1} N-AGA were added at both apical and basal sides of the cells for 48 hours. For sweet taste receptor functional assay, after differentiation, cells were grown in glucose and glutamine free DMEM supplemented with 10% dialyzed FCS. Inhibitor, 1 mmol.L^{-1} lactisole (Sigma Aldrich, Saint Quentin, France) and substrate 25 mmol.L^{-1} fructose, were added during the last 48 hours of culture at both apical and basal poles.

When grown on porous support, cell viability and cell monolayer integrity after treatments were estimated by measure of the transepithelial electrical resistance (TEER), which is a witness of tight junction integrity and of ion pump function in cell membranes (Grasset et al., 1984).

Immunofluorescence: Differentiated Caco-2/TC7 cells grown on filters were fixed with 4% Paraformaldehyde (Sigma Aldrich, Saint Quentin, France), permeabilized with 0.2% Triton (Sigma Aldrich, Saint Quentin, France) and labeled with antibodies targeting T1R2 (T-20: sc-22456) and T1R3 (N-20: sc-22458) (Santa Cruz, Biotechnology, Tebu France). Images were produced by confocal microscopy (Zeiss LSM510 software).

Human GLUT2, GLUT5 and sucrase-isomaltase promoter constructs: Caco-2/TC7 cells were transfected with the following promoter regions inserted into p205 plasmid driving the reporter gene luciferase (Rodolosse et al., 1996) : -1100/+300 of the hGLUT2 promoter (generous gift of GI Bell, University of Chicago, Chicago, IL, USA), –3600/+60 of the hSI promoter (Rodolosse et al., 1997) and -2500/+21 of the hGLUT5 promoter (Mahraoui et al., 1994). Populations of stably transfected cells were established. Protein assays were made with the BCA kit (Pierce, Interchim, Montluçon, France). Maximal variations of protein concentrations between different cell cultures and different culture conditions were below 20%. Luciferase activities were measured using the Luciferase assay kit (Promega, Charbonnières les Bains, France) in a Lumat LB9501 luminometer (Berthold Detection System, Pforzheim, Germany). Results were expressed as relative light units $RLU.sec^{-1}.\mu g^{-1}$ protein.

EGFP-GLUT2-loop and -C-terminus peptide constructs: The coding regions of the intracellular loop between transmembrane domain 6 and 7 (amino acids 237-301) and the C-terminus GLUT2 domain (amino acids 481-521) of rat GLUT2 were cloned in frame with EGFP in pEGFP-C (Clontech BD Biosciences, le Pont de Claix, France), as previously described (Guillemain et al., 2000). The EGFP-GLUT2 domain cassettes were placed downstream the SV40 promoter of pGL3 (Promega, Charbonnières les Bains, France), allowing the expression of EGFP-GLUT2 domains in differentiated Caco-2/TC7 cells. Stably transfected EGFP positive cells were established and sorted by FACS (Epics Altra, Beckman Coulter, Roissy, France). The cells were secondarily and transiently transfected with the hGLUT2 promoter using the lipofectin transfection kit (Life Technologies, Cergy Pontoise, France).

Messenger RNA: Total RNA from the jejunum of mice or from Caco2/TC7 cells were extracted using TriReagent (MRC, Interchim, Montluçon, France). Mouse and human

GLUT2, L-Pyruvate kinase (L-PK), Glucose-6-phosphatase (G-6-Pase), Sucrase-Isomaltase (SI) and human SGLT1 and GLUT5 mRNAs were quantified by reverse transcription and real-time PCR using the Light-Cycler System according to the manufacturer's procedures (Roche Molecular Biochemicals, Meylan, France) as previously described (Gouyon et al., 2003a; Guillemain et al., 2002). The primers used were for hGLUT5 forward 5'-TCTCCTTGCAAACGTAGATGG-3'and reverse 5'-GAAGAAGGGCAGCAGAAGG-3', for hSGLT1 forward 5'-TGGCAATCACTGCCCTTTA-3'and reverse 5'-TGCAAGGTGTCCGTGTAAAT-3' and for hADRP forward 5'-GTGAGATGGCAGAGAACGGTGTG- 3'and reverse 5'-TGCCCCTTTGGTCTTGTCCA-3'. All primer pairs amplified a single amplicon as indicated by the unique melting temperature of the PCR product. Moreover, we verify the size and specificity of the amplicon by restriction enzyme analysis and electrophoresis. The large ribosomal protein L19 was used as a control gene since its expression level varied by less than 20% in all the culture conditions applied to the cells. Results were expressed as ratios of gene mRNA over L19 mRNA levels. Northern blots were performed to measure mouse intestinal GLUT5 and SGLT1 mRNA. Density analyses (Gel Analyst 3.02 software) were expressed as the ratio of mRNA to 18S rRNA. For human taste receptors identification, primers were selected using published data (Rozengurt et al., 2006) or predicted sequences available in the NCBI database: NM_152232 for T1R2, NM_152228 for T1R3, and XM_001129050, XM_294370 and X_M939789 for Gustducin. T1R2: forward 5'-GTATGAAGTGAAGGTGATAGGC-3'and reverse 5'-GGGTAGACCACCCTCTTGG-3'; T1R3: forward 5'-CAAGTTCTTCAGCTTCTTCCTC-3' and reverse 5'-GTACATGTTCTCCAGGAGCTGC-3'; Gustducin: forward 5'-GCCAAATACATTTGAAGATGCAGG-3' and reverse 5'- GCACTTCTGGGATTTACATAATC-3'. Note that for T1R2 and T1R3 nested primers were also used. Nested T1R2: forward 5'-TGCGCTTCGCGGTGGAGG-3'and reverse 5'-CAGCCGAGGAGGCTGTGC-3'; nested T1R3: forward 5'-GGTCAGCTACGGTGCTAGC-3'and reverse 5'-AGCCTGAGGCGTTGCACTG-3'.

Sugar transepithelial transfer: The glucose or fructose content of the apical and basal culture media was assayed with an enzymatic assay kit (Sigma Aldrich, Saint Quentin, France). The non-specific transpithelial transfer of sugar across Caco-2/TC7 cells grown on porous support was measured using (1-^{3}H NEN) L-glucose. L-glucose transfer was lower than 1.5 % of the D-isomers (3 independent experiments, data not shown) indicating that epithelia were tight. Using fructose media helped to distinguish fructose and glucose transporting GLUTs.

Statistics : All statistical analyses were made using ANOVA and student T tests (PRISM software).

RESULTS

Regulation of intestinal genes by dietary sugars

In vivo experiments were first conducted to determine if glucose and fructose employ distinct signalling pathways to induce sugar-regulated genes. We focused our study on sugar transporters (GLUT5, GLUT2, SGLT1) and sugar metabolism enzymes (SI and L-PK). As previously observed for GLUT2 (Gouyon et al., 2003a), glucose- or fructose-rich diets efficiently stimulated the mRNA accumulation of these sugar-regulated genes in the jejunal mucosa of wild-type (WT) mice (Figure 1). SGLT1, SI and L-PK mRNA increased to similar levels under both glucose and fructose dietary regimens (Figure 1). On the other hand, GLUT5 mRNA increased in mice fed fructose- but not glucose-rich diets (compare Figure 1A to 1B), in agreement with previous studies (Burant and Saxena, 1994; Gouyon et al., 2003b) indicating that cells discriminate glucose and fructose signals to regulate the expression of GLUT5. As glucose and fructose are both catabolized through glycolysis in enterocytes, metabolic signalling via glycolysis cannot fully account for the differential effect. We used GLUT2-null mice to document the contribution of this glucose/fructose transporter to the regulation of sugar-sensitive genes. The mRNA levels were similar for the different genes analysed in GLUT2-null and WT mice fed low carbohydrate diets. GLUT2-null and WT mice exhibited indistinguishable increases of GLUT5 mRNA in response to fructose-rich diet. Similarly, the small but significant induction of SGLT1 by sugar-rich diets was similar in GLUT2-null and WT mice. By contrast, SI and L-PK mRNA inductions were not observed or were dramatically reduced in GLUT2-null mice. Therefore, a GLUT2-dependent signalling pathway is required to obtain SI and L-PK gene regulation by dietary glucose and fructose. Interestingly, feeding GLUT2-null mice with fructose produced a partial accumulation of L-PK mRNA (Figure 1B), indicating that fructose and glucose signalling pathways can differ in the intestine. These *in vivo* data indicate that enterocytes exploit several sugar signalling pathways to adapt the expression of sugar target genes to the dietary environment.

Effect of sugars on sensitive genes in Caco-2/TC7 enterocytes

Enterocyte detection of dietary sugars could occur at their apical membranes exposed to the intestinal lumen content or their basolateral membranes exposed to the blood. To assess the sugar detection capacity of each of these membranes in a controlled manner, we used differentiated human colon carcinoma, Caco-2/TC7 cells that display the morphological characteristics and functional properties of intestinal absorptive cells (Delie and Rubas, 1997).

Caco-2/TC7 cells cultured on solid support have only their apical poles in contact with culture media, whereas cultures grown on porous support permit sugar provision to either apical or basolateral membranes. This system was thus employed to differentially expose enterocytes to dietary sugars and to determine polarity in sugar detection and signalling.

As shown in Figure 2, fructose stimulated hGLUT5 promoter activity by 2 fold in Caco-2/TC7 cells regardless of whether they were grown on solid or porous support. By contrast, neither glucose nor fructose could stimulate the hGLUT2 promoter activity in cells grown on solid support (Figure 2), although the same hGLUT2 promoter fragment has been shown capable of being activated by glucose in pancreatic MIN6 cells (Cassany et al., 2004). However, a net stimulation of hGLUT2 promoter activity was observed in Caco-2/TC7 cells grown on porous support in agreement with the capacity of dietary sugars to induce GLUT2 *in vivo*. Thus growing enterocytes on porous support with their basal poles capable of detecting sugars unmasks polarity in sugar signalling toward GLUT2 gene regulation.

We took advantage of the restricted localization of SI at the apical membrane of enterocytes to further study the mechanisms of polarity in sugar detection and signalling. The addition of sucrose into apical medium stimulated hSI promoter activity significantly (Figure 2), but no activation was obtained when sucrose addition was restricted to media exposed to the basolateral membrane. Furthermore, sucrose cannot be simply hydrolyzed to fructose and glucose at the basolateral membrane as it lacks SI activity. These data also indicate that epithelial tight junctions were functional. Importantly, the glucose and fructose moieties of sucrose stimulated the hSI promoter activity irrespective of the compartment of addition (Figure 2). Thus, enterocytes growing on porous support display polarity in their responses to dietary sugars.

When administered independently to the apical or basal compartments, glucose and fructose concentrations equilibrated between the apical and basolateral media within 12 hours (Figure 3A). Therefore, in our culture conditions, polarized sugar signals could only be detected within 12 hours (i.e. before sugar equilibration is achieved). We measured the time course of hGLUT2 promoter activity induced by sugar (Figure 3B, left panel) and unfortunately, the addition of sugar required 48 hours to maximally stimulate the hGLUT2 promoter and little to no stimulation was observed at 12 hours (Figure 3B, left panel). Accordingly, the addition of glucose to either pole of the cell for 48h (i.e. long after sugar equilibration is achieved), produced a similar stimulation of the hGLUT2 promoter (not shown).

By contrast, 12 hours of sugar removal were sufficient to significantly decrease hGLUT2 promoter activity and near basal levels were obtained after 24 hours (Figure 3B right panel). After 12 hours, hGLUT2 promoter activity was significantly higher in cells deprived of apical sugar but supplied with glucose at the basal pole (Figure 3C) whereas strict apical provision of sugar did not activate the hGLUT2 promoter significantly within the same time course (Figure 3C). This is congruent with the inability of sugars to stimulate the hGLUT2 promoter when the basolateral membranes of Caco-2/TC7 cells are attached on solid support (Figure 2). These results suggested that basolateral glucose provides a sugar signal able to maintain the transcription of hGLUT2. Moreover, the difference in hGLUT2 promoter stimulation between strictly basal versus basal and apical sugar supply might reveal a role for sugar metabolic fluxes in these signalling processes. Lastly, we found the activation of the hGLUT2 promoter exhibited saturation at 20 $mmol.L^{-1}$ (data not shown) in accordance with the kinetic parameters of the GLUT2 transporter (Gould et al., 1991), the main sugar transporter in the basolateral membrane of enterocytes (Cheeseman and O'Neill, 1998).

Sugar sensitive genes are induced by metabolism

Sugar metabolism is an essential component of the regulation of sugar sensitive genes in hepatocytes and adipocytes. We investigated the contribution of metabolic signalling to the expression of selected glucose-sensitive genes in enterocytes, as GLUT2, L-PK and glucose-6-phosphatase (G-6-Pase). The glucose analogue, 3-O-methylglucose (3OMG) is a substrate for SGLT1 and GLUT2 that can be transported but not metabolized. Provision of 3OMG to Caco-2/TC7 cells did not increase hGLUT2 promoter activity even when cells were cultured on porous support (Figure 4A), suggesting a metabolic requirement for the induction of this glucose-sensitive gene. However, the structural difference introduced by glucose methylation might not be suitable for all sugar-signalling machineries. The contribution of metabolism was thus further analyzed by inhibiting glucose phosphorylation into glucose-6-phosphate. N-acetylglucosamine (N-AGA) is an efficient inhibitor of hexokinases, it did not affect the expression of genes that were not regulated by sugar like Adipose Differentiation-Related Protein (ADRP) (Figure 4B) or Cyclophilin A (not shown). N-AGA completely prevented glucose-dependent G-6-Pase mRNA increase as expected from earlier reports demonstrating that G-6-Pase promoter activity is strictly regulated by glucose metabolism (Massillon, 2001) (Figure 4B). However, N-AGA only partly prevented GLUT2 and L-PK mRNA increases (Figure 4B). These data confirmed that an active metabolic pathway is necessary to sustain a proper transcriptional response to glucose in enterocytes. Interestingly, N-AGA also reduced

GLUT2 mRNA levels in the absence of added extracellular glucose, indicating that metabolism contributes to basal promoter activity due to the low levels of glucose provided by the serum (Figure 4B). However, inhibition of glucose metabolism did not abolish sugar-dependent increases of GLUT2 and L-PK mRNAs, suggesting either the existence of some additional sugar signalling pathways or an incomplete block of hexokinases by N-AGA.

Additional sugar signalling pathways exist in enterocytes

GLUT2-loop mediated pathway

Both our data in mouse jejunal mucosa and our data in Caco-2/TC7 cells show that metabolism is necessary but not sufficient to fully explain the response of sugar sensitive genes to glucose or fructose in enterocytes. Therefore, the possibility that GLUT2 could function as a sugar detector and thereby mediate sugar signalling was tested. Indeed, in mhAT3F hepatoma cells, we previously showed that GLUT2 is capable of triggering glucose-dependent signalling by interacting with the karyopherin alpha2 protein through its large cytoplasmic loop between transmembrane domain 6 and 7 (Guillemain et al., 2000). We here asked if different GLUT2 constructs could functionally alter dietary sugar sensing. Two intracellular domains of GLUT2 were expressed in Caco-2/TC7 enterocytes, to test their effect on sugar-sensitive gene expression. Cells that stably expressed EGFP-GLUT2-loop, EGFP-GLUT2-Cterminus or EGFP alone, were obtained by cell sorting and cells were selected for similar levels of transgene expression (Figure 5A, upper panel). As expected, EGFP-GLUT2-loop exhibited a nuclear location whereas EGFP or EGFP-GLUT2-Cterminus did not present any specific location (Figure 5A, lower panel). In cells expressing EGFP alone, as in non-transfected cells, fructose and glucose increased endogenous hGLUT2 mRNA levels by 4- and 3-fold respectively (compare Figure 5B and 2). EGFP-GLUT2-Cterminus expression did not significantly change endogenous hGLUT2 mRNA accumulation in response to sugar (Figure 5B). By contrast, EGFP-GLUT2-loop expression reduced endogenous hGLUT2 mRNA accumulation by 60% in response to fructose and by 90% in response to glucose as compared to controls (Figure 5B). Similar results were obtained for hL-PK mRNA(Figure 5C) and hSI mRNA (not shown) in response to glucose.

To determine whether these strong inhibitions were due to reduced transcription, we measured transiently transfected hGLUT2 promoter activity in the stable lines expressing EGFP-GLUT2-loop and EGFP-GLUT2-Cterminus (Figure 5D). In accordance with the mRNA accumulation data, EGFP alone and EGFP-GLUT2-Cterminus did not interfere with

the fructose-dependent stimulation of hGLUT2 promoter activity, whereas EGFP-GLUT2-loop partially inhibited it.

We assumed that the remaining stimulations of GLUT2 and L-PK observed in EGFP-GLUT2-loop expressing cells were due to a sugar metabolism pathway. N-AGA could further inhibit endogenous GLUT2 mRNA accumulation in EGFP-GLUT2-loop cells (Figure 5E), as well as L-PK mRNA accumulation. These results indicate that both metabolic and GLUT2-dependent signalling are necessary for the regulation of sugar sensitive genes.

Sweet-taste receptor mediated sugar signalling

As sweet-taste receptors of the GPCR family have been implicated in fructose and sucrose signalling, we questioned the putative role of T1R sweet taste receptors in enterocytes challenged by fructose. Functional assays were performed in differentiated Caco-2/TC7 enterocytes cultured in the presence of T1R3 ligands. Lactisole, a T1R3 inhibitor, decreased the stimulation by fructose of human SGLT1, GLUT5, and L-PK mRNA expression (Figure 6A), underlining the involvement of T1R3 in fructose signalling. By contrast, neither GLUT2 mRNA levels (Figure 6A) nor hGLUT2 promoter activity (not shown) were changed by lactisole, suggesting that T1R3 did not control GLUT2 expression. By RT-PCR, the T1R3 receptor expression was revealed, as expected from functional assays. Its cognate G protein, Gustducin ($G_{\alpha gust}$) also known as GNAT3 (Guanine nucleotide binding protein, alpha transducing 3) was identified (Figure 6B). Moreover, neither T1R3 nor Gustducin mRNA expressions were modified by glucose or fructose (Figure 6B). T1R2 and T1R3 proteins were recovered in plasma membranes of fully differentiated Caco-2/TC7 enterocytes by confocal analysis (Figure 6C). We could locate the sweet-taste receptors mainly at the basolateral membranes of enterocyte cells (Figure 6C).

DISCUSSION

In this study we show the convergence of several sugar signalling pathways on the regulation of intestinal sugar-sensitive target genes. In addition to the better understood metabolic signalling mechanisms, we identified GLUT2 and GPCRs to be important sugar sensing signalling initiators governing enterocyte gene regulation. We identified a pathway strictly requiring GLUT2, since it was absent in GLUT2-null mice and could be blocked in dominant-negative fashion in cells expressing a large amount of the cytoplasmic loop domain of GLUT2. Particularly important was finding the polar nature of GLUT2 sugar sensing and signalling from the basolateral and not the apical enterocyte membrane. Thus as enterocytic GLUT2 senses hyperglycaemia it could further increase sugar delivery to the blood by the up-regulation of sugar transport and metabolism genes.

The GLUT2 pathway had different and complementary effects compared to the well-studied metabolic pathways with respect to the genes they regulated, suggesting differences in the molecular mechanics of these two pathways. In mammalian cells, sugar metabolites, including glucose-6-phosphate or xylulose-5-phosphate can mediate the regulation of sugar-sensitive gene expression (Vaulont et al., 2000). Both metabolites are thought to accumulate in the cells and to convey sugar signals to the transcription machinery. In hepatocytes, xylulose-5-phosphate has been proposed to activate protein phosphatase 2A (Nishimura and Uyeda, 1995), which in turn would dephosphorylate a transcription factor named carbohydrate response element binding protein (ChREBP). ChREBP would then be transported into the nucleus to stimulate the transcription of glucose-sensitive genes (Uyeda et al., 2002). ChREBP is very abundant in the intestine (Uyeda et al., 2002), but it might not be the only transcription factor to regulate glucose-sensitive genes. Indeed, all glucose-sensitive promoters do not contain ChREBP binding sites (ChoRE, carbohydrate response element). For instance, in hepatocytes, the GLUT2 promoter rather binds SREBP-1C (sterol response element binding protein) on a sterol responsive element (Im et al., 2005). SREBP-1C also stimulates the transcription of lipogenic genes in response to glucose and insulin (Wang et al., 2003). In addition, in pancreatic cells, SREBP-1C was emphasized over ChREBP in the activation of the transcription of GLUT2 and Insulin Receptor Substrate by glucose (Wang et al., 2005). The metabolite that activates SREBP-1C has not yet been identified, and the specific role of SREBP-1C in glucose-induced gene expression in the intestine is unknown. Sugar transport and metabolism in enterocytes rely on the abundance and kinetic parameters

of several transporters. Among them, the high Km and high Vmax transporter GLUT2 is abundant in the basolateral membranes allowing a large influx of sugars and providing abundant fuel for the metabolic pathway in enterocytes.

In hepatocytes, GLUT2 not only triggers a metabolic-signal but it also triggers a protein signalling cascade (Guillemain et al., 2002). In Caco-2/TC7 cells, high expression of the large cytoplasmic loop between transmembrane domain 6 and 7 of GLUT2 inhibited the stimulation by sugars of GLUT2 and L-PK and its expression was restricted to the nucleus as in hepatoma cells. Since the inhibition by the GLUT2-loop was partial and could be further inhibited by hexokinase inhibition, we concluded that the metabolic and GLUT2 pathways are both required in enterocytes. The nuclear importer karyopherin alpha2 can bind to the GLUT2 loop, furthermore the rate of its nuclear shuttling is regulated by glucose in hepatoma cells (Cassany et al., 2004). This nuclear importer is overexpressed in diabetic kidney suggesting that its expression is regulated by sugar (Kohler et al., 2001). Moreover, in Caco-2/TC7 cells unmasking their basal pole, karyopherin alpha2 expression was recovered in cells that exhibited GLUT2-triggered sugar signalling pathway (not shown). Karyopherin alpha2 imports proteins into the nucleus, and could trigger the ultimate steps of the effect of sugars on the transcription machinery. The convergence of and the cross-talk between metabolic- and GLUT2-pathways appeared to be an important way sugar-sensitive genes can be regulated. Transcriptions factors involved in glucose sensing such as ChREBP (Uyeda et al., 2002) and liver X receptor LXR (Mitro et al., 2007) are known to be translocated to the nucleus in response to extracellular sugar (Helleboid-Chapman et al., 2006; Kawaguchi et al., 2001). We speculate that if these transcription factors bind to the karyopherin alpha2 then it will constitute the ultimate step of the GLUT2-triggered sugar signalling pathway.

GPCRs offer additional possibilities for sugar detection at the plasma membrane and for sugar-dependent signal transduction. Indeed, GPCRs that bind fructose and sucrose have been shown to transduce sugar signals in the tongue and several taste receptors have been identified in an enteroendocrine cell line (Nelson et al., 2001; Rozengurt, 2006). Studies in knockout mice support a role of T1R2/T1R3 heterodimers and T1R3 homodimers in sweet preferences (Zhao et al., 2003). In enterocytes, the use of lactisole (an inhibitor of T1R3) suggests that T1R-dependent signalling is involved in the regulation of some fructose-stimulated genes. The signal transduction mechanisms used by these G protein-coupled sweet receptors are less clear (Wettschureck and Offermanns, 2005). The Gα Gustducin, mainly expressed in taste cells, is believed to be able to couple receptors to phosphodiesterase resulting in a decrease of

cyclic nucleotide levels (Ozeck et al., 2004). However, studies also implicate that a receptor-activated G_S can activate adenylyl cyclase to generate cAMP (Striem et al., 1989). Sugar provision to enterocytes increases intracellular cAMP levels suggesting that the regulation of SGLT1 (Dyer et al., 2003) and GLUT5 (Gouyon et al., 2003b; Mahraoui et al., 1994; Mesonero et al., 1995) rely on adenylate cyclase/PKA pathways. Moreover, we report here the presence of the T1R3 cognate Gα Gustducin in Caco-2/TC7 cells. We speculate that sweet taste receptor pathways contribute to fructose effects on GLUT5 and L-PK mRNA accumulations, which were still detected in GLUT2-null mice.

Finally, we present here evidence that the sugar signal can be polarized in enterocytes. Whereas the apical supply of fructose elicited stimulation of GLUT5 mRNA expression, the basolateral supply of sugars generated a transcriptional response of GLUT2. *In vivo*, in neonatal rat intestine, plasma fructose was shown to regulate the expression of GLUT2 (Cui et al., 2003). However, the experimental design *in vivo* did not distinguish between direct effects of plasma fructose from secondary signals. Our data in Caco-2/TC7 cells support the idea that sugars can directly signal from the basolateral membrane. Thus detectors located at the basolateral membrane trigger a polarized sugar signal. Here, we show that T1R2 and T1R3 are both located in basolateral membranes of differentiated enterocytes. Moreover, GLUT2 itself is the main sugar transporter in the basolateral membrane of enterocytes (Cheeseman and O'Neill, 1998). The physiological role of the stimulation by basolateral sugar provision of the expression of glucose transporters is unclear, however it could be a thrifty mechanism well suited to an environment in which nutrient supplies are rare and need to be fully absorbed. This mechanism however could become a threat for organisms facing a nutritionally rich environment that would permanently stimulate sugar uptake capacities in the intestine. In the diabetic state, hyperglycaemia is maintained during interprandial periods and this sustains the transcription of sugar target genes via a basolateral signal and might contribute to glucose toxicity in the long term. At the apical membrane, SGLT1 transports glucose and galactose and GLUT5 transports fructose, but these transporters are saturated rapidly as sugar concentrations rise. After a sugar-rich meal, GLUT2 transiently translocates into the apical membrane and improves the transport capacity of the intestine (Kellett and Brot-Laroche, 2005). Hence, GLUT2, depending on its relative abundance in the apical and basolateral membranes, is likely to trigger sugar signals from the blood or the intestinal lumen content. Whereas GLUT2 may be transiently expressed in the apical enterocyte membrane, it is permanently high in the basolateral membrane, and may thereby determine the polarity of sugar detection.

At least three sugar-signalling pathways have now been identified suggesting the capacity for fine tuning of intestinal sugar delivery. The molecular identification of sugar-detectors embedded in the enterocyte plasma membrane provides new therapeutic targets and strategies toward controlling intestinal sugar delivery for the improved health of patients suffering hyperglyceamic episodes.

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6

FIGURE LEGENDS

Figure 1: Dietary sugars regulate intestinal genes in mice

Messenger RNA samples were from jejunal mucosa of wild-type (white bars) or GLUT2-null (black bars) mice fed a protein-, glucose- or fructose-rich diet for 5 days. Northern blots (mSGLT1, mGLUT5) and RT-PCR (mL-PK, mSI) were performed. Results represent the average +/- SEM from at least 6 mice and are presented as the ratio of mRNA levels in sugar over low carbohydrate (protein) dietary conditions. mRNA levels under a protein diet were set at 1 (dotted line). (*) Statistical significance ($P < 0.05$) comparing sugar- to protein-fed mice; (#) Statistical significance ($P < 0.05$) comparing wild-type and GLUT2 null mice; (NS) Non significant.

Figure 2: Polarized sugar supply affects the expression of sugar sensitive genes in human enterocytes

Caco-2/TC7 cells stably expressing either hGLUT5-, hGLUT2- or hSI-promoter luciferase constructs, were cultured on either solid or porous support for 17 days. After differentiation of the cells, culture media were without sugar addition (white bars) or supplemented with either 25 mmole.L^{-1} glucose (G, black bars), 25 mmole.L^{-1} fructose (F, hatched bars), 12.5 mmole.L^{-1} sucrose (S, large hatched bars), or a 12.5 mmole.L^{-1} equimolar, glucose and fructose mix (FG, grey hatched bars). Sugars were added to the apical and/or basal compartments for 4 days as indicated. Luciferase activities in RLU.sec^{-1}.μg^{-1} protein were normalized to basal as measured in cells cultured in media without sugar addition for 4 days. (*) Statistical significance ($P < 0.05$) compared to low sugar conditions; (NS) Non significant; (NA) Non-applicable for cells cultured on solid support.

Figure 3: Basolateral sugar sensing regulates hGLUT2 promoter activity

(A) Time course of sugar transfer across Caco-2/TC7 cells relative to the compartment of sugar application (left panel apical supply, right panel basolateral supply). Apical sugar is denoted by filled diamonds, basolateral sugar is denoted by open squares. Sugar decrease in the compartment of origin is denoted by dotted lines while sugar appearance in the opposite compartment is indicated by a solid line. Sugar concentrations reached equilibrium within 12 hours. For panels B and C, Caco-2/TC7 cells stably expressing an hGLUT2-promoter luciferase construct were used. (B, left panel) Time course of hGLUT2-promoter activity in cells apically and basolaterally provided with 25 mmole.L^{-1} glucose (filled squares; G25) or

fructose (filled circles, F25) after having been cultured for 4 days without sugar addition (Low sugar (LS) is denoted by open diamonds). (B, right panel) Time course of hGLUT2-promoter activity in cells deprived of glucose in apical and basolateral compartments. (C) hGLUT2-promoter activity in cells after glucose-deprivation either in the apical, basal or apical and basal compartments for 12 hours. Note that the promoter is sensitive to basally applied glucose. Luciferase activities in $RLU.sec^{-1}.\mu g^{-1}$ protein were normalized to basal amounts measured in cells cultured in media without sugar addition for 4 days (B, left panel) or in cells cultured in media containing glucose (B, right panel and C). (*) Statistical significance ($P < 0.05$) comparing to t=0h; (NS) Non significant.

Figure 4: Metabolic pathways control sugar-sensitive gene expression

(A) Differentiated Caco-2/TC7 cells stably expressing the hGLUT2-promoter luciferase construct were cultured on porous support, either without sugar addition (white bars) or with 25 $mmole.L^{-1}$ glucose (black bars) or with 25 $mmole.L^{-1}$ 3-O-Methylglucose (3OMG, hatched bar) for 48 hours. Luciferase activities $RLU.sec^{-1}.\mu g^{-1}$ protein were normalized to values without glucose addition and are represented as the mean +/- SEM (n=6). (B) RT-PCR analysis of hADRP, hGLUT2, hL-PK and hG-6-Pase mRNA in differentiated Caco-2/TC7 cells cultured on porous support in the presence or absence of 25 $mmole.L^{-1}$ glucose and/or 250 $mmole.L^{-1}$ N-acetylglucosamine (N-AGA) for 48 hours. hADRP/L19, hGLUT2/L19, hL-PK/L19 and hG-6-Pase/L19 ratios were normalized to the control level in cells cultured without added sugar. Results are representative of three independent experiments and are represented as means +/- SEM (n=3). (*) Statistical significance ($P < 0.05$) compared to control; (#) Statistical significance ($P< 0.05$) compared N-AGA treated cells; (NS) Non significant.

Figure 5: GLUT2 is a sugar detector controlling gene expression

(A) Upper panel: FACS profiles of Caco-2/TC7 cells untransfected (parental) and stably expressing EGFP, EGFP-GLUT2-Cterminus or EGFP-GLUT2-loop constructs. Lower panel: Epifluorescence of EGFP, EGFP-GLUT2-Cterminus or EGFP-GLUT2-loop proteins in Caco-2/TC7 transfected cells. Toto-3 is a nuclear marker. Endogenous hGLUT2 (B) and hL-PK (C) mRNA expression is shown from control cells (untransfected or EGFP expressing cells) or from cells expressing EGFP-GLUT2 domains, cultured on porous support in glucose (black bars, B and C) or fructose (hatched bars, B) for 4 days. hGLUT2/L19 and hL-PK/L19 mRNA

ratios were normalized to control levels reached in cells in media without sugar addition (dotted line). (D) Caco-2/TC7 cells stably expressing EGFP-GLUT2 domains were secondarily and transiently transfected with the hGLUT2 promoter luciferase construct. Luciferase activities ($RLU.sec^{-1}.\mu g^{-1}$ protein) measured in 4 days fructose stimulated cells were normalized to conditions without sugar addition (dotted line). (E) Endogenous human GLUT2 mRNA abundance in the presence of 250 $mmole.L^{-1}$ N-acetylglucosamine (N-AGA) in control or in EGFP-GLUT2-loop Caco-2/TC7 cells for 48 hours. hGLUT2/L19 mRNA ratios were normalized to mRNA levels in cells without sugar addition. Data were expressed as means +/- SEM (n=6). (*) Statistical significance ($P < 0.05$) compared to control conditions; (#) statistical significance ($P< 0.05$) comparing control and EGFP-GLUT2-loop cells; (NS) non significant.

Figure 6: Identification of GPCR-mediated sugar signalling in enterocytes

(A) Human GLUT2, GLUT5, L-PK and SGLT1 mRNA abundance was measured in Caco-2/TC7 cells using quantitative RT-PCR. Fully differentiated cells were maintained for 2 days in sugar free medium (white bars), before challenge with 25 $mmole.L^{-1}$ fructose (F, hatched bars) or 25 $mmole.L^{-1}$ fructose and 1 $mmole.L^{-1}$ lactisole (F+Lact, grey and hatched bars) for 48 hours. Messengers/L19 mRNA ratios were normalized to basal levels in cells in media without sugar addition. Data were expressed as the mean +/- SEM of 2 independent experiments. (*) Statistical significance ($P < 0.05$) compared to control conditions; (#) Statistical significance ($P < 0.01$) comparing F and F+Lact; (NS) non significant. (B) Semi-quantitative RT-PCR of T1R3 sweet taste receptor and $G\alpha_{gust}$ in differentiated Caco-2/TC7 cells cultured without sugar addition (Low Sugar, LS) or in media supplemented with 25 $mmole.L^{-1}$ fructose (F), 25 $mmole.L^{-1}$ glucose (G) for 4 days. (C) Confocal microscopy analysis of filter-grown differentiated Caco-2/TC7 cells labelled with T1R2 and T1R3 antibodies. Serial Z sections of Caco-2/TC7 cells displayed similar pattern of basolateral location for T1R2 and T1R3 receptors. White bars represent 10µm.